Mechanisms Controlling Day/Night Changes in CAM Tissue Volume, With a Focus on the Kansas Cactus, Opuntia macrorhiza

By

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Submitted to the graduate degree program in the Department of Ecology and Evolutionary Biology and the Graduate Faculty of the University of Kansas in partial fulfillment of the requirements for the degree of Master of Arts.

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Date Defended: On 20 September 2017

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Date Approved: On 5 October 2017

Abstract

Day/night changes in organs volume of Crassulacean Acid Metabolism (CAM) plants have been observed. The objective of this study is to determine the most important mechanism that controls day/night changes of organ thickness in CAM plants. In this study, day/night changes in organ volume and morning and evening acidities of organs were measured. The focus was on the CAM species *Opuntia macrorhiza* under different conditions. Mechanisms that may explain these day/night changes in organs volume of CAM plant could be day/night changes in internal CO_2 pressure, day/night changes in water content or day/night changes in temperature. Pervious study suggested that day/night changes could be due to internal CO_2 pressure inside tissues or water content. This study confirmed these two mechanisms and has added a new variable which is day/night changes in temperature.

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Introduction

Photosynthesis Pathways:

Although all plants photosynthesize using the same light reactions to convert light energy to chemical energy, plants can fix CO_2 using one of three photosynthetic pathways: C_3, C_4 or CAM, (Aragón,2013). The C_3 photosynthetic pathway is the most common photosynthetic pathway on earth (Jessica et al, 2006); it is a widespread photosynthetic pathway in both optimal and stressful environments (Chahdoura, 2015). This pathway occurs in plants that use only the Calvin cycle to capture carbon dioxide to produce six carbon sugars. C_3 plants usually grow where the light level, temperature, and water availability are moderate.

C_4 plants are usually found in hot and dry areas with high levels of light. Unlike C_3 plants, C_4 plants have a special anatomy, Kranz anatomy which is critical for operation of the C_4 pathway, in which CO_2 is concentrated around the enzyme Rubisco, allowing a high rate of photosynthesis, even under stress (Jessica et al, 2006). In Kranz anatomy, vascular tissues and mesophyll cells are surrounded by bundle sheath cells. The Kranz anatomy helps a C_4 plant to avoid photorespiration. Photorespiration occurs when [CO_2] inside the leaf is low. Kranz anatomy provides a location in which CO_2 can be concentrated around RuBisCo (Jessica et al, 2006).

In order to increase water use efficiency, CAM plants close their stomata during the day (Bowes, 2008). The stomata open during the night, which allows for uptake of CO2, while minimizing the evaporative loss of water vapor. Therefore, the amount of water lost on a 24-hour basis is very low, relative to C_4 and C_3 plants. This provides CAM plants with a strong selective advantage in arid environments. Moreover, CAM plants are usually succulent which is another adaptation to drought stress (Horner, 2012). In CAM plants at night, CO_2 is utilized by PEP carboxylase, and is converted to the mild acid malate that is stored in the vacuole. CAM plants have a large vacuole that can occupy up to 95% of the cell volume (Davis, 2014). During the day,

the malate is released from the vacuole and is decarboxylated in the cytoplasm. The CO_2 that is released into the intracellular space is subsequently used by Rubisco, the enzyme that is responsible for fixing carbon dioxide in the Calvin Cycle to make sugar (Hsu,2006; Kenyon et al., 1981)

Thickness and CAM Tissue

Most CAM plants have thick, succulent vegetative organs. Two previous studies have found that the thickness of CAM organs increases in the day and decreases during the night (Chen & Black, 1983; Winter & Andrew, 2012). In contrast, leaves of the C_3 plant *Brassica rapa* (Brassicaceae) were found to be thicker during the dark period (Chen & Black, 1983). Chen and Black claimed that the day/night changes in CAM organs could be due to CO_2 pressure and stomatal resistance. The high concentration of CO_2 in the intercellular spaces during the day might create a high gas pressure, thereby increasing the thickness of the CAM plant organs (Chen & Black, 1983). During night periods CO_2 is absorbed, which might decrease the volume of the CAM organs (Chen & Black, 1983). Chen and Black also suggested that other factors may be involved in regulating CAM organ volume during the day and night periods. There have been no other studies that have examined the causal mechanisms of day/night changes in CAM organ thicknesses.

Hypotheses

Because the causes of CAM changes in organ volume remain unknown, it was the purpose of the current research to determine the mechanisms underlying the observed fluctuations in stem thickness of CAM plants. In the present study, five hypotheses are presented that might explain the day/night fluctuations in thickness of CAM organs:

1) Day/night changes in organs is related to partial CO_2 pressure:

• CO_2 pressure is high during the day and low in the night.

2) Day/night changes in organs are due to total water content in the vegetative organs:

• The water content in tissues during the day is higher than it is throughout the night.

3) The possible interaction of extremely thick organs (succulence) with other factors:

• Succulence can play a role in fluctuations in volume of tissues since the volume water content inside tissues is, by definitions, very high.

4) Day/night changes are due to changes in ambient temperature:

• High temperature during the day and low temperature during the night.

Rationale hypotheses

1) During the day when the stomata are closed the internally generated CO_2 fills out intercellular spaces in CAM organs, causing an expanded organ volume. In contrast, at night the CO_2 in the intercellular spaces is utilized by PEP carboxylase and the reduced gas pressure causes organs the organs to shrink.
2) During the night, stomata are open and water is lost, so the tissues shrink. The water potential will decrease as a result of losing water.
3) If succulence is a main factor in causing day/night changes CAM volume, a C_3 succulent should show the same day/night changes as CAM succulent.

4) During the day, the low water potential (from the night) draws water other part of the plant or soil the tissues expand. The high daytime temperature directly expands the volume of organs, and the low nighttime temperature will decrease the volume of organs.

Materials and Methods

Study species

Opuntia macrorhiza is a CAM species. *Opuntia macrorhiza* plants were collected from 20 km south of Yates Center, Kansas at 37°53'06" N and 95°50'47" W. They were then rooted and grown in pots 22cm in height and 17cm in diameter. The soil used was 50% standard glasshouse soil mix and 50% sand. The plants were grown under the following environmental conditions in the greenhouse at the University of Kansas: 1000 µmol m^{-2} s^{-1} maximum photosynthetic photon flux density (PPFD; natural photoperiod), day/night air temperature ranges of 27– 36/15– 26°C, and day/night vapor pressure deficit (VPD) ranges of 1.3–3.0/ 0.7–1.6 kPa. 500 µmol m^{-2} s^{-1} PPFD (during the day for all species); 30.0/24.9°C day/night temperatures and 2.8/1.8 kPa day/night VPD at 500 µmol m^{-2} s^{-1} PPFD for all species, (Martin & Woerner, 1999; Vonwillert & Martin,2000).

Also, *Hoya carnosa* and *Stapelia grandiflora* were measured under control conditions as other CAM species. They were purchased from local nursery, kept in the Green house for two years.

The plants were watered two to three times per week and fertilized once every two weeks (stock material: 18% total N, 18% available P_2O_5, 18% soluble K_2O, and trace elements). After five years of growth, five plants of *Opuntia macrorhiza* were placed in a growth chamber under the following conditions:

An average temperature of 33.2 C day and 21.4 C night, average chamber humidity of 19% during the day and 32% during the night. The photoperiod was 12 hours.

Measuring stem thickness

An electronic thickness measuring device (Burster Präzisionmeß technik GmbH, Gernsbach, Germany) was used to measure fluctuations in stem thickness absorption by attached stems of *Opuntia macrorhiza* and leaves of *P. scandens* (CAM), *P. obtusifolia* (C_3). in the growth chamber (environmental conditions: 300–350 µmol m^{-2} s^{-1} PPFD for 15 h, 27/188C day/night air temperatures, and 2.2/0.7 kPa day/night vpd). (8) This device was capable of measuring these fluctuations in stem thickness with a resolution of 1 µm. A steel rod, 2 mm in diameter and weighing 2.1 g, rested vertically on the adaxial surface of an attached, horizontal stem or leaf (Figs. 1a, b)

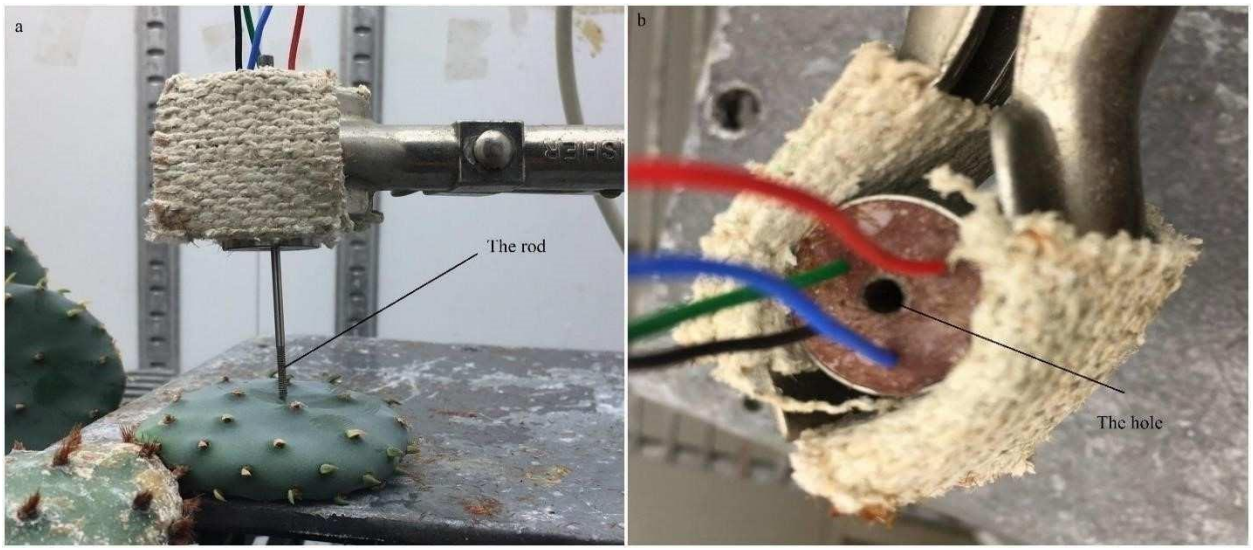

Fig. 1 (a) The rod can move up and down through a hole. (b) The hole that is connected to reading screen.

The upper end of the rod could move easily in a cylindrical hole in the device in which an electrical oscillator created a magnetic field. Movements of the rod, due to fluctuations in stem or leaf thickness, disrupted this field; such disruptions were transduced and calibrated to yield data in µm. Fluctuations were linear throughout the range of the instrument (0–5.08 mm). The attached pad (stem) of cactus was placed horizontally on expandable metal platform (Big Jack Precision Scientific Co. Jaxline Big Lab Jack.) Height: 6 in. Overall dimensions: 8 in. L x 6

in. W x 6 in. H, Chicago, USA) that can be easily adjusted. The rod of the instrument was placed in the middle the stem. The distance between the edge of the pad and where the rod was placed was 53.6 mm.

Acidities

The tissue samples were taken in the morning of the second day at 9:30 am when the acid should be high and at the beginning of the night normally at 8:00 pm of the same day when the acid should be low. The acid samples were taken from the same pad used for thickness measured by the rod. At each collection time, the sample was cut in the form of an Isosceles triangle. The base of the triangle was the edge of the cactus pad. The opposing angle of the triangle was where the rod was placed. The samples were put in a plastic bag in the refrigerator for two days before calibration. Then, the samples were taken out and let them in a cylinder for 20 minutes. After that, every sample was ground with a mortar and pestle with 40 mils of water. The acidity of the solution was determined by titration a tissue solution to pH 7.0 with 0.01N NaOH, using was PH meter (Fisher Scientific™ accumet™ AB15 Basic and BioBasic™ pH/mV/°C Meters).

Treatments

Control treatment

Initially, five individuals of *O. macrorhiza* (average thickness 12.74 mm) were exposed to standard conditions, and thickness was measured over the course of at least 48h.

The constant light treatment

The same five individuals of *O. macrorhiza* were exposed to a constant light for 48 hours (300–350 µmol m^{-2} s^{-1} PPFD.) All other conditions were as same as control.

Low concentration CO2 treatment

Three individuals of *O. macrorhiza* were exposed to a low concentration of CO_2 (312315ppm)

by placing soda lime in the chamber. All other conditions were the same as the control treatment. CO_2 level and the light intensity were measured by a LI-6400XT Portable Photosynthesis System (LI-COR, INC. Lincoln, NE.)

The constant temperature treatment

The five Individuals of *O. macrorhiza* were placed in the growth chamber and the temperature was set to be the day temperature 33.2 C. for both the dark and light period. All other conditions were the same as the control treatment.

High concentration of CO2 treatment

The same three individuals of *O. macrorhiza* that were exposed to the low concentration of CO_2 were exposed to a high concentration of CO_2. Dry ice was put inside the growth chamber and used to release CO_2 (approximately 1523ppm) for 48 hours. The measurements of CO_2 were taken on the second day of the experiment by the thickness measurement device. All other conditions were the same as the control treatment.

Humidity treatment

The five individuals of *O. macrorhiza* were exposed to high relative humidity for two days. The relative humidity was approximately 98% for 48 hours. Chamber humidity was increased by wetting the surfaces inside the growth chamber. Four tanks (16 liter) for each, three wet towels (27 inches x 52 inches), and small sink (4 liter) were used to increase the humidity inside growth chamber. All other conditions were the same as the control treatment.

Succulence effect

Both *P. obtusifolia* (C_3) and *P. scandens* (CAM) are succulent. They display a similar growth habit and both are epiphytes. Also, they grow in the same location. The thickness and acidity of *P. obtusifolia* and *P. scandens* were measured under control conditions. The sample

size of each was three. The rod was put in the middle of the leaf. The distance between the edge and the rod was about 34.5mm.

Experimental rationale:

1. Constant temperature: If day/night changes in temperature is important, constant temperature will prevent day/night changes in organs thickness. Previous study showed that constant temperature inhibited CO_2 uptake during the night, (Martin & Siedow, 1981). Increasing temperature may decrease CO_2 uptake during the night, and reducing malate content in the morning (Lin, Qin, et al., 2006) Also, high temperature during the night might inhibit malate uptake by vacuole (Behzadipour, et al., 1998).
2. High CO_2: Increased atmospheric CO_2 should increase the amount of malate stored overnight (Weiss et al., 2009). Also, increasing CO_2 would decrease water uptake due to stomatal closure (Cui & Nobel, 1994; Ceusters et al., 2008).
3. Low CO_2: Decreasing CO_2 reduces the malate formation at night, (Cote et al., 1989); however, low [CO_2] decreases stomatal resistance which might counteract the latter effect of low CO_2 (Cockburn, 1979).
4. High humidity: In this experiment, transpiration was stopped by increasing the relative humidity to test the effect day/night changes in water content on day/night changes in thicknesses. So, by increasing the relative humidity, day/night changes in thicknesses were not expected. According to Martin & Siedow (1981), the high water content inside tissues might inhibit CO_2 uptake. However, Fanourakis et al. (2017) found that there is no effect of increasing relative humidity on stomatal conductance in *O. macrorhiza*.

5. Constant light: Martin & Siedow (1981) reported that in constant illumination experiment CO_2 exchange pattern was similar to normal day/night light condition. However, constant light decreases of internal CO_2 in the morning (Kluge et al, 1981).

Results and Discussions

Effect of Control Conditions on Organ Thickness of Three Species of CAM Plants

The thicknesses of CAM tissues fluctuated during the day/night period for *O. macrorhiza* (Fig. 2a). The thickness of the CAM organs (stems) at mid-day was greater than the thickness at mid-night (Fig. 2b). The amount of acid was higher in the morning than at the beginning of night (Fig. 2c). Also, thicknesses and acidities of organs from additional CAM species (*Hoya carnosa* and *Stapelia grandiflora)* were measured (fig. 3). The thickness of the CAM organs (leaves) of *Hoya carnosa* varied between mid-day and mid-night (fig. 3a). The acidity of CAM organs of *Hoya carnosa* in the morning is higher than in the evening (fig. 3b). The stems of *Stapelia grandiflora* at mid-day are thicker than at mid-night (fig. 3c). The acidity of leaves of *Stapelia grandiflora* did not vary between the morning and evening (fig. 3d).

The increase in the early morning might be due to the high pressure of CO2. The stem thickness of Opuntia macrorhiza increased in the day after the temperature increased and the CO2 that was released from the malate. Then, at the beginning of the night a fast decrease occurs as a result of decreased CO2 pressure. Also, the water content may have an effect on organ thickness. During the night, the stomata are open and losing water then organs shrink. However, during the day, the stomata are closed and organs draw water from soil and expand. Moreover, temperature may affect the organ thickness. The temperature during the day is higher than during the night. So, the high temperature during the day may expand the organs, whereas the low temperature during the night shrinks the organ thickness.

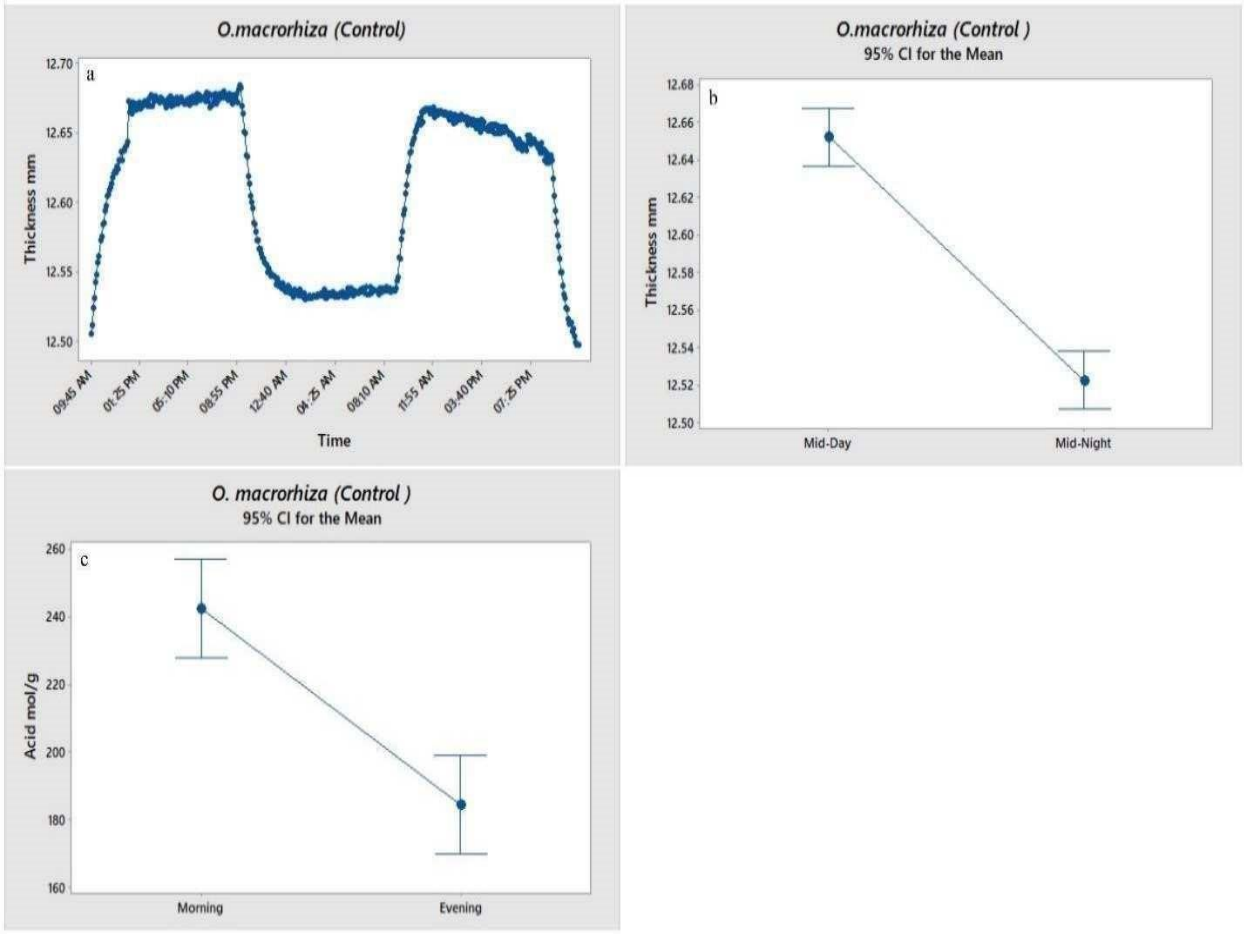

Fig. 2 (a) Day/night changes in stem of *O. macrorhiza* under control conditions, (b) Stem thickness at midday and mid-night (difference is significant at p value 0.05), (c) Stem acidity in the morning and stem acidity at the evening (difference is significant at p value 0.05).

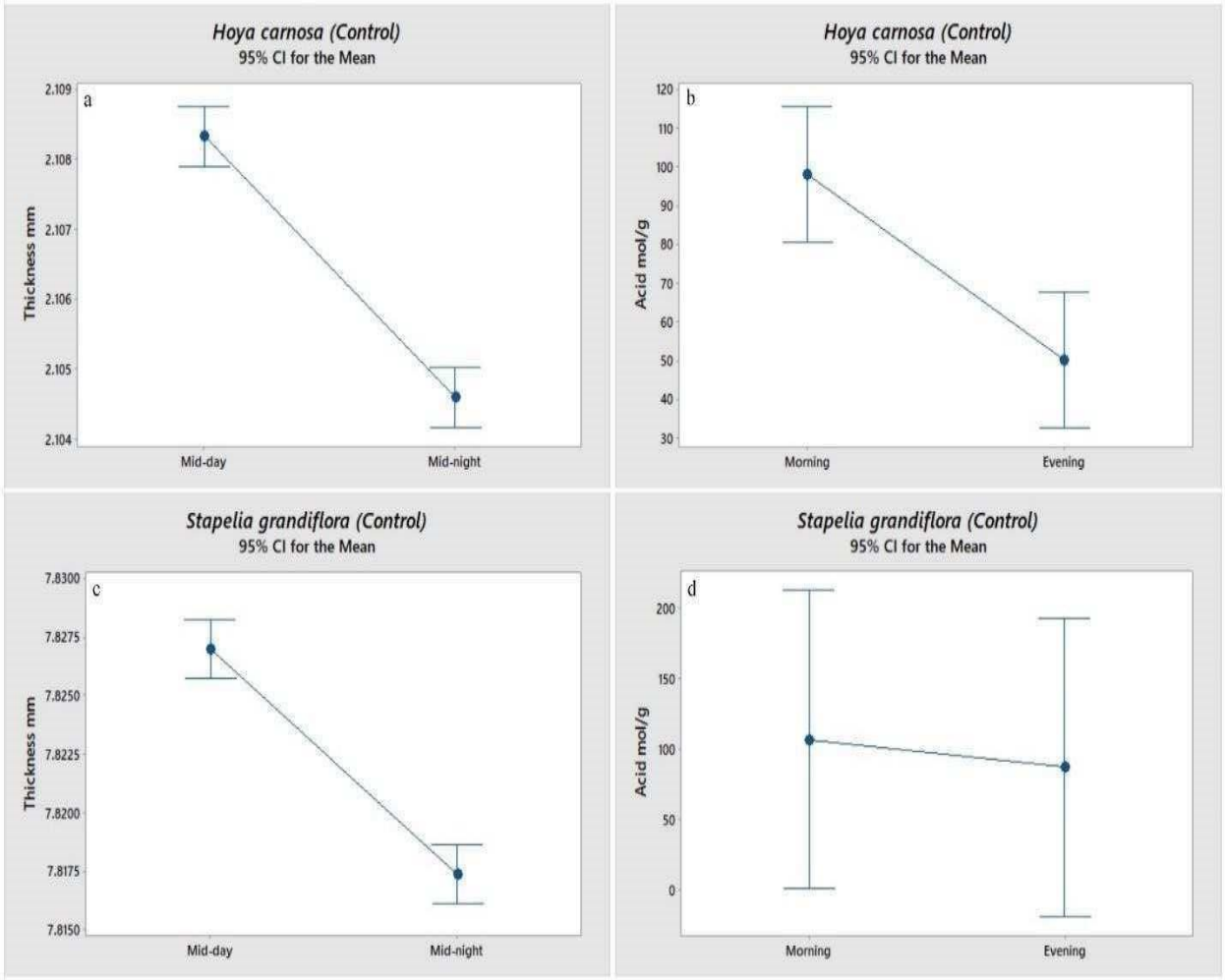

Fig. 3 (a) Leaf thickness of *Hoya carnosa* at mid-day and mid-night (difference is significant at p value 0.05), (c) leaves acidity of *Hoya carnosa* in the morning and leaves acidity at the evening (difference is significant at p value 0.05), (c) leaves thickness of *Stapelia grandiflora* at mid-day and mid-night (difference is significant at p value 0.05), (d) leaves acidity of *Stapelia grandiflora* in the morning and leaves acidity at the evening (the difference is not significant at p value).

Effect of Constant Day temperature on organ thickness of *Opuntia macrorhiza*

The temperature inside the chamber was set to be same as day temperatures for 48 hours with no change for other conditions (see materials and methods). Organs of *O. macrorhiza* did not vary between midday and midnight (Figs. 4a, b). Even though increasing temperature during the night may inhibit CO_2 uptake, (Martin & Siedow, 1981), the organ acidity was higher in the morning than evening (Fig. 4c)

demonstrating that the constant temperature experiment did not affect the CAM. The temperature may not be high enough to inhibit CO_2 uptake.

High temperature increases the volume of water that occupy up to 95% of cell's volume. Therefore, the pressure of water vapor can increase with increased temperatures. Therefore, the thickness of organ may increase (Corey, 1990). Even though the plant is still doing CAM, the CAM organs did not vary between day and night. Also, there is no change in water content since the stomata are open during the night and capture CO_2 by PEP carboxylase.

Effect of constant Light on organ thickness of *Opuntia macrorhiza*

In this experiment, light was on for 48 hours in the growth chamber with no changes in other conditions. The thickness of organs at midday was greater than the thickness at midnight but did not cycle in thickness as under control conditions (Figs. 5a, b; compare two data points at 12am in 5a). Acidity did not vary between midday and midnight (Fig. 5c).

Martin & Siedow claimed that constant light may not affect pattern of CO_2 uptake, however, we found that the plant did not do CAM under constant light. This difference between expectation and our results could be due to the difference between species that were used in these studies. The stomata of *O. macrorhiza* are closed during the day Therefore, increased light exposure may increase the use CO_2 by Rubisco, but not PEP because Rubisco is activated by light, whereas PEP is inactivated (Haynes, 1975). Because the amount of CO_2 released from malate is light-dependent, the gas (CO_2) pressure increases. Therefore, the fluctuations in the organs of CAM plants cannot be explained by CAM because there was no CAM. Also, there was no changes in day/night temperature in this treatment, so we can explain the lack of day/night changes in thickness by lack of day/night changes in temperature.

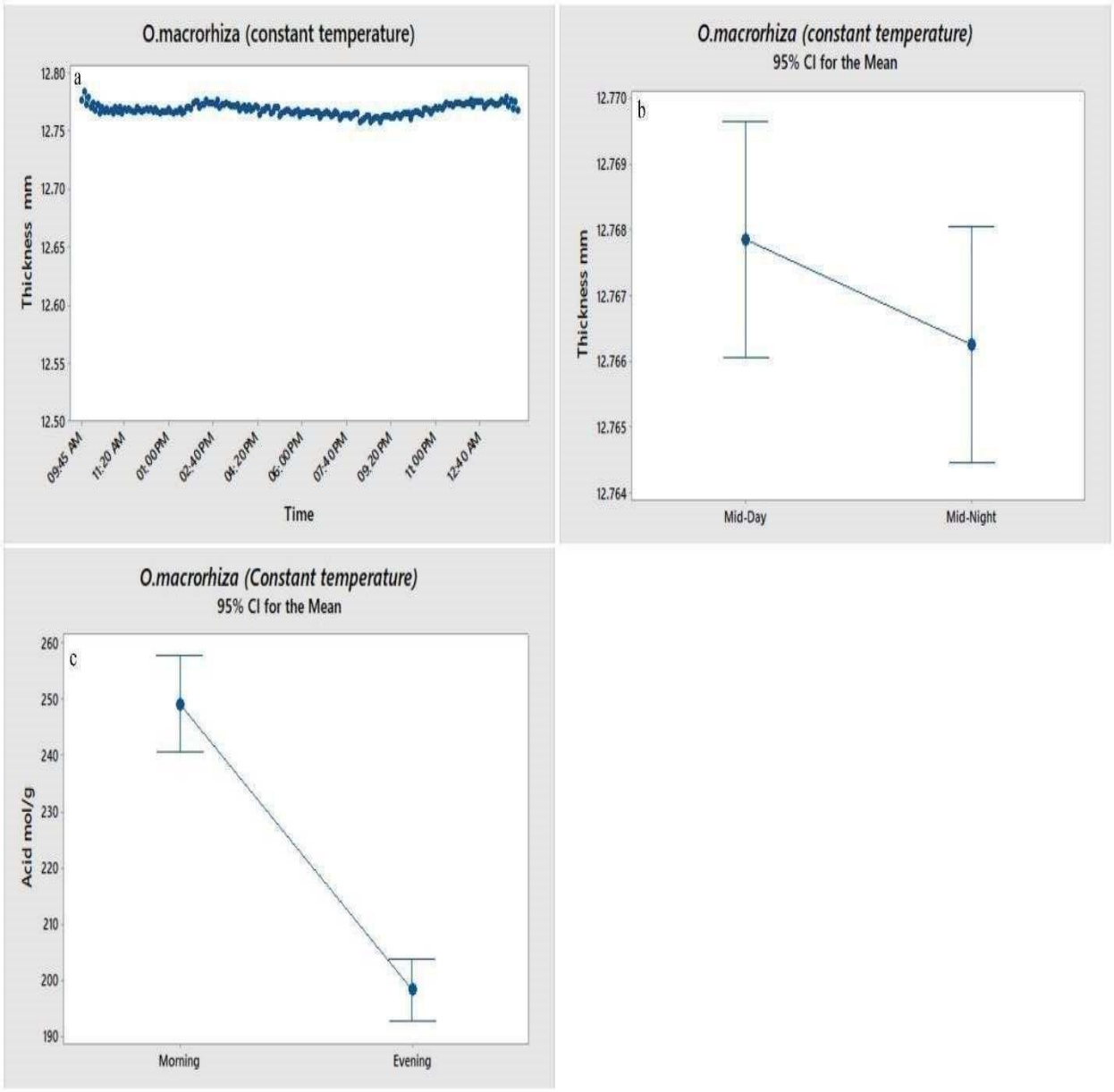

Fig. 4 (a) Day/night changes in stem of *O. macrorhiza* under constant temperature, (b) Stem thickness at mid-day and mid-night (difference is not significant, p value is more than 0.05), (c) Stem acidity in the morning and stem acidity at the evening (difference is significant at p value 0.05).

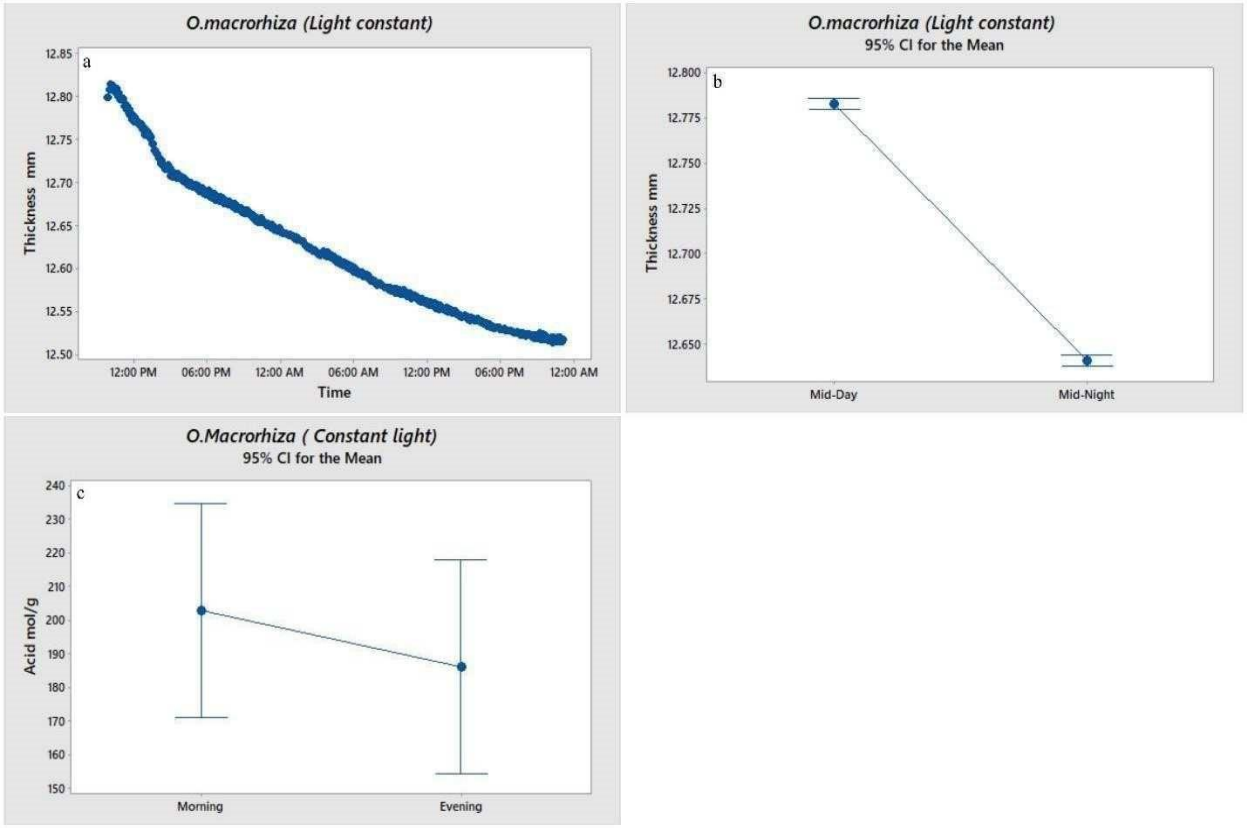

Fig. 5 (a) Day/night changes in stem of *O. macrorhiza* under constant light, (b) Stem thickness at midday and midnight (difference is significant at p value is 0.05), (c) Stem acidity in the morning and stem acidity at the evening (difference is not significant, p value is more than 0.05).

Effect of low concentration of CO_2 on organ thickness of *Opuntia macrorhiza*

The concentration of CO_2 was low (312-315 ppm) with no changes in other conditions. Organ thickness of *O. macrorhiza* did not vary between the midnight and the midday (Figs. 6a, b). Even though low [CO_2] might decrease the stomatal resistance which might counteract the effect of low CO_2, (Cockburn,1979), the acidities did not vary between morning and evening (Fig. 6c).

With a low concentration of CO_2 available relative to the control treatment, PEP carboxylase would make less malate, (Kenyon,1981). Therefore, the CO_2 that is released during the day from malate would be low, and the thickness decreases as a result (Lack &Evans, 2005).

Even though the temperature during the day was higher than at night, the organs did not vary between midday and midnight. Also, the humidity was as same as control treatment, so the water content could not explain the changes in CAM organs.

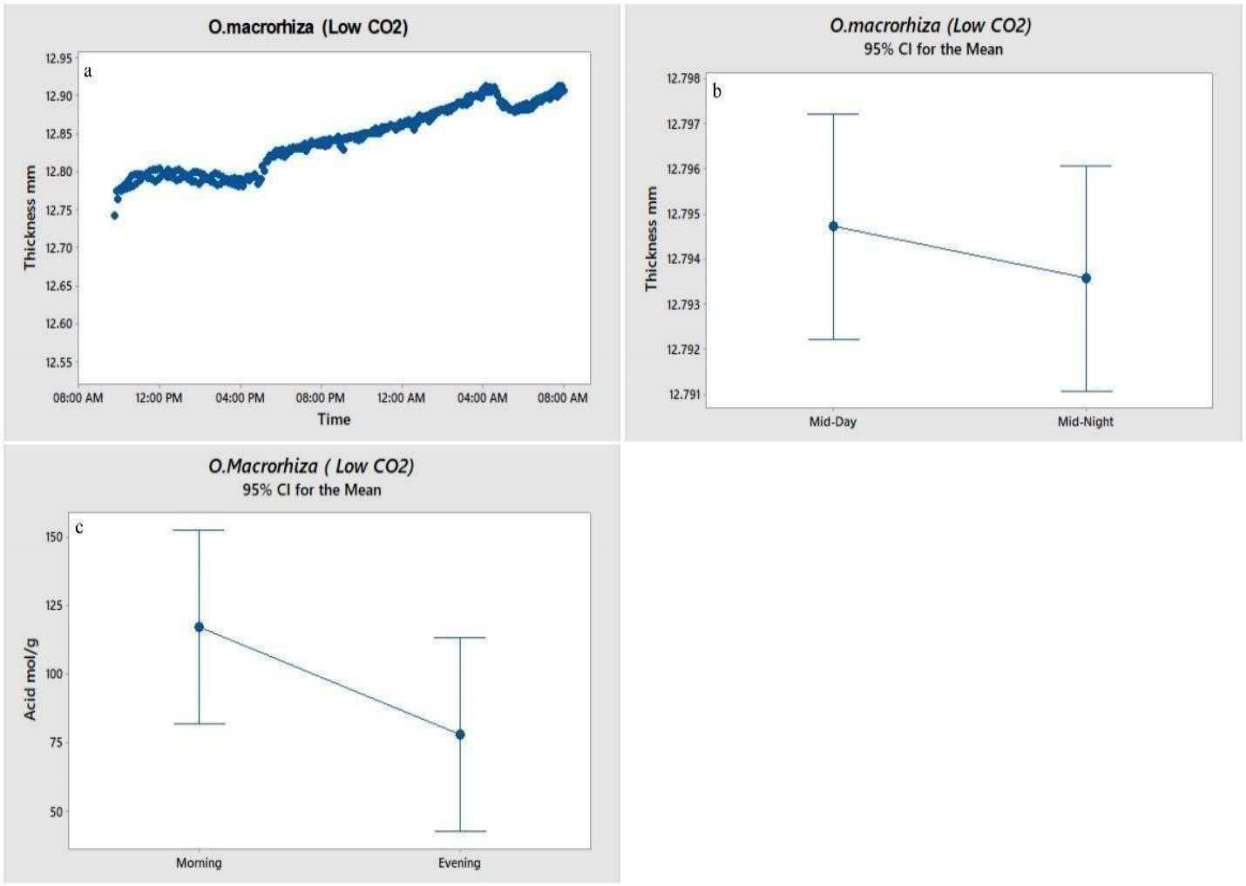

Fig. 6 (a) Day/night changes in stem thickness of *O. macrorhiza* under low [CO_2], (b) Stem thickness at mid-day and mid-night (difference is not significant, p value is more than 0.05), (c) Stem acidity in the morning and stem acidity at the evening (difference is not significant, p value is more than 0.05).

The effect of high concentration of CO_2 on organ thickness of *Opuntia macrorhiza*

On the other hand, the concentration of CO_2 was increased by the release of CO_2 inside the chamber and was higher than normal (1523 ppm) with no changes in other conditions. With high CO_2 available, relative to control, PEP carboxylase would make more acid (Weiss et al., 2009). However, organs thickness did not vary

between midday and midnight (Figs. 7a, b). Also, the acidities of morning and evening did not vary (Fig. 7c). We expected a high amount of acid, (Weiss et al., 2009). However, high [CO_2] may cause stomata closure and prevent plant from doing CAM (Kenyon,1981).

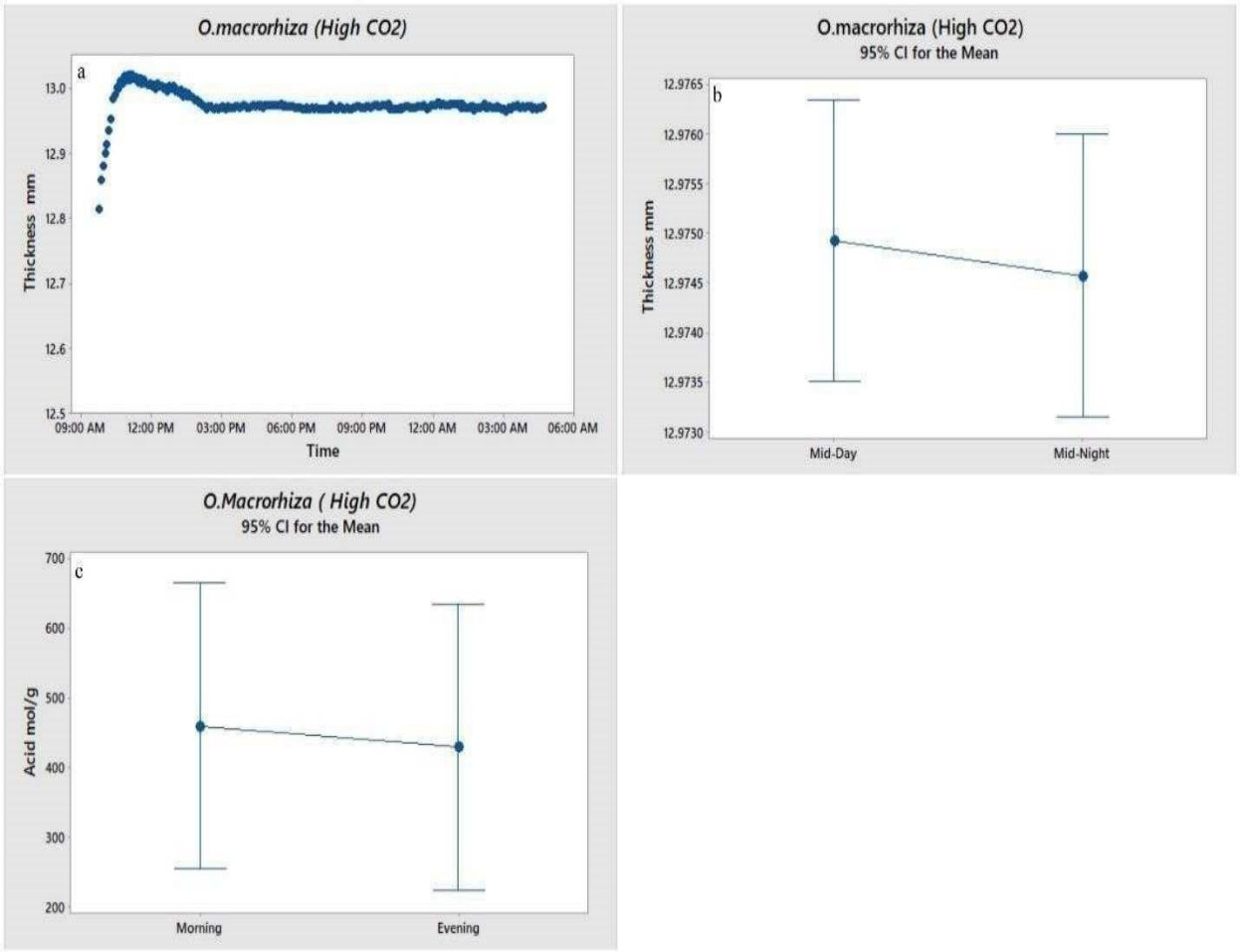

Fig. 7 (a) Day/night changes in stem thickness of *O. macrorhiza* under high [CO_2], (b) Stem thickness at mid-day and mid-night (difference is not significant, p value is more than 0.05), (c) Stem acidity in the morning and stem acidity at the evening (difference is not significant, p value is more than 0.05).

As a result of stomatal closure during the day, the gas pressure will affect the thickness during the light period. The gas pressure will increase while the concentration of CO_2 increases when the CO_2 is released from malate thought the day. The high amount of CO_2 results in making more acid at night. During the subsequent daytime, the acid is moved to the cytoplasm

where it is decarboxylated and releases CO_2 that increases the pressure and increases organ thickness since the stomata are closed.

However, it is not clear whether water content affects the thickness of CAM organs because the stomata may be closed during the night due to high concentration of CO_2 inside organs (Kenyon, 1981). Even though the temperature varied between day and night, the organs' thickness did not vary.

The Humidity effect on organ thickness of *Opuntia macrorhiza*

In this experiment, all conditions were under control conditions except the humidity was about 98%. The organs did not vary between mid-day and mid-night (Figs. 8a, b), but the plant was still doing CAM (Fig. 8c) as expected; because there is no effect of increasing relative humidity on stomatal conductance (Fanourakis et al., 2017).

Because there is no transpiration occurring due to the high humidity around the stomata, the plant will lose less water from its organs (James, 1975). This would explain the higher thickness of CAM plant tissues during the night. Even though the plant was doing CAM, the CAM could not explain the changes because organ thickness did not vary between midday and midnight. Also, even though the temperature varied between day and night, the organ thickness did not vary between day and night.

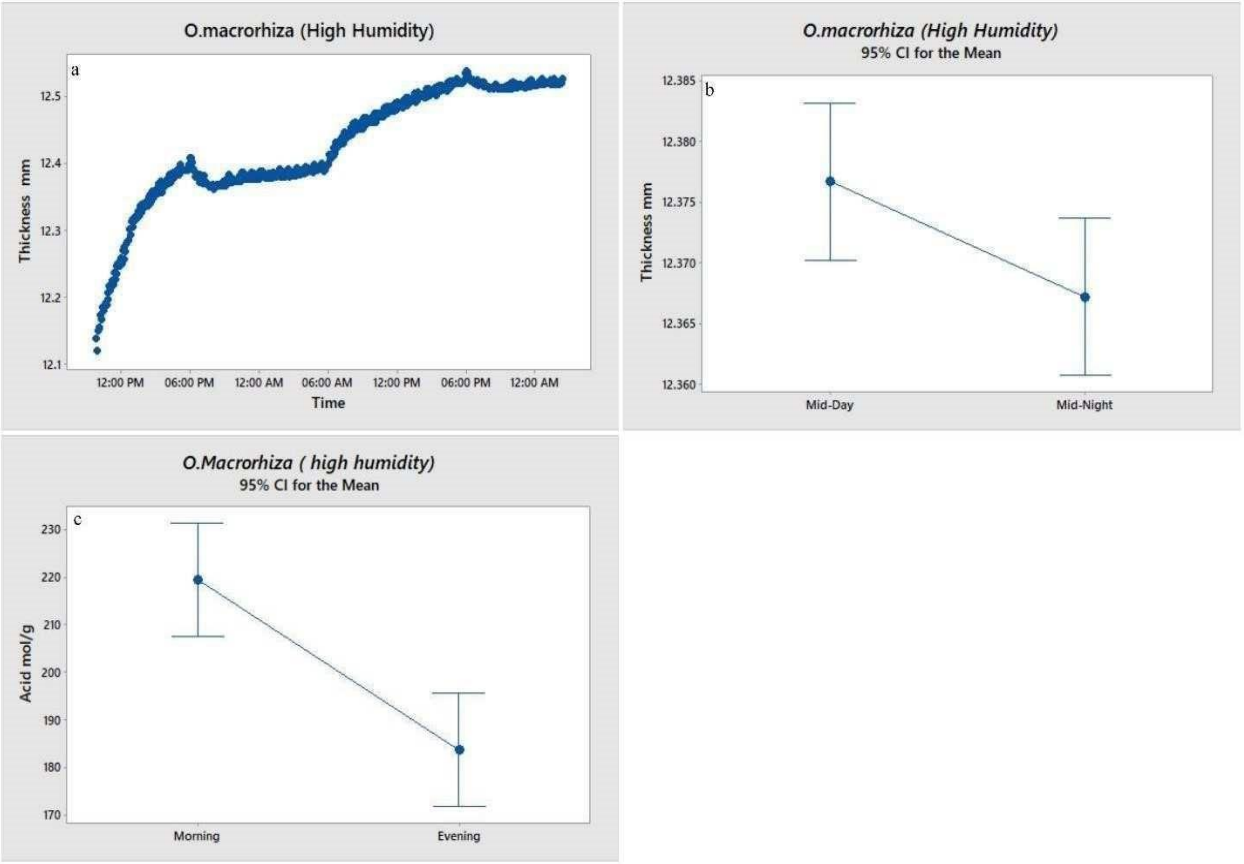

Fig. 8 (a) Day/night changes in stem of *O. macrorhiza* under high humidity at night, (b) Stem thickness at mid-day and mid-night (difference is not significant, p value is more than 0.05), (c) Stem acidity in the morning and stem acidity at the evening (difference is significant at p value 0.05).

The Succulence effect on organ thickness of *Opuntia macrorhiza*

Opuntia macrorhiza is a succulent CAM plant, and it was tested under control conditions. The purpose of the final experiment was to test whether the succulent is responsible for changes on the CAM plant's organs. To test the specific role of succulence, two other succulent species were grown under control conditions and results compared to results from *O. macrorhiza*. They were *Peperomia obtusifolia* (C_3) and *Peperomia scandens* (CAM), (Gibeaut, 1989). The thickness of *Peperomia scandens* organs varied between day and night (Figs. 9a, b). Also, organs acidity varied between day and night (Fig. 9c). The average thickness of the leaf

of *Peperomia scandens*' is 2.45 mm. The other leaf succulent plant that was measured is *Peperomia obtusifolia.* It is a C_3 plant and the average thicknesses of the leaf is 2.33 mm. The thicknesses and acidity of organs of *peperomia obtusifolia* did not vary between midday and midnight (Figs. 9c, d, f). Based on the results of succulence experiment, it is clear that changes in thickness are not solely the result of organ succulence because both *Peperomia scandens* and *peperomia obtusifolia* were succulent but only one species (CAM) exhibited day/night changes thickness.

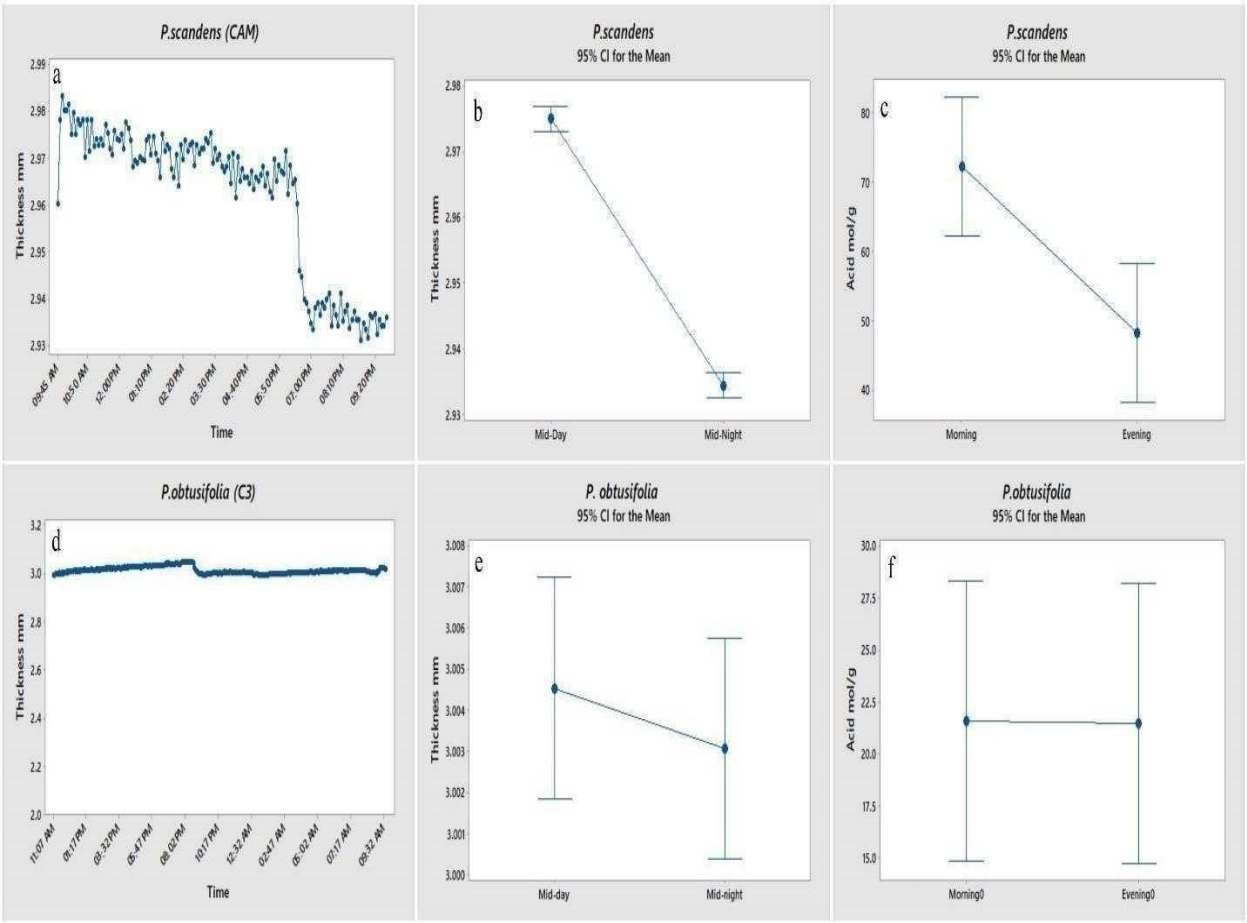

Fig. 9 (a) Day/night changes in leaf thickness of *P. scandens* under control, (b) Leaf thickness at midday and midnight (difference is significant at p value 0.05), (c) Leaf acidity in the morning and leaf acidity at the evening (difference is significant at p value 0.05). (d) Day/night changes in leaf thickness of *P. obtusifolia* under control, (e) Leaf thickness at mid-day and mid-night (difference is not significant, p value is more than 0.05), (c) Leaf acidity in the morning and leaf acidity at the evening (difference is not significant, p value is more than 0.05)

Conclusion

Fluctuation in thickness in CAM plant has been considered as a unique phenomenon that has not been studied very well. This study tested four hypotheses to set appropriate explanations. CAM could be the main reason for this changing in thickness of stem. CAM allows CO_2 to be absorbed due to stomata opening during the night, which results in CO_2 being fixed by PEP into malate. During the subsequent light period, stomata are closed and the CO_2 that is released by malate when the light period takes place. The CO_2 inside the tissues will increase pressure and results in an increase in volume and thicker organs at midday. Factors associated with CAM can explain the variation in organ thickness in the control, low [CO_2], and high [CO_2] treatments, but it could not explain constant day temperature and high humidity treatments when there was no variation despite doing CAM. A high temperature will expand organs, so temperature has an effect on organ thickness due to the expansion that results from increasing the temperature. The constant day temperature could explain control, and constant day temperature treatment, but it could not explain high humidity, low [CO_2], and high [CO_2] treatments. So, we may conclude that the CAM and constant day temperature are the most effective factors affecting organ thickness in this experiment (Fig. 10).

Experiments	ΔDay/night changes in thickness	Δ in Acid	Mechanisms that can explain day/ night changes in thickness
Control with standard conditions	**Yes**	**Yes**	ΔW, ΔT, ΔDay/night CO_2 pressure.
Constant Day/night temperature	**No**	**Yes**	ΔT
Constant Day/night light	**Yes**	**No**	ΔT
Low [CO_2] at night	**No**	**No**	ΔW, ΔDay/night CO_2 pressure.
Hi [CO_2] at night	**No**	**No**	ΔW, ΔDay/night CO_2 pressure.
High humidity at night	**No**	**Yes**	ΔW

Fig. 10

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